Universidad de Córdoba

Programa de Doctorado:

Ingeniería Agraria, Agroalimentaria, Forestal y de Desarrollo Rural Sostenible

TESIS DOCTORAL

Integrated control of avocado white root rot through biological and chemical methods

Control integrado de la podredumbre blanca del aguacate mediante métodos biológicos y químicos

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Instituto de Agricultura Sostenible – Consejo Superior de Investigaciones Científicas

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TITULO: *Integrated control of avocado white root rot through biological and chemical methods*

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Memoria de Tesis Doctoral presentada por JUAN MANUEL ARJONA LÓPEZ, Ingeniero Agrónomo, para optar al grado de Doctor por la Universidad de Córdoba con la mención de Doctorado Internacional.

Dirigida por:

Carlos José López Herrera

Científico Titular

Dpto. Protección de Cultivos

Instituto de Agricultura Sostenible (CSIC)

Córdoba, Diciembre 2019

TÍTULO DE LA TESIS: CONTROL INTEGRADO DE LA PODREDUMBRE BLANCA DEL AGUACATE MEDIANTE MÉTODOS BIOLÓGICOS Y QUÍMICOS

DOCTORANDO/A: JUAN MANUEL ARJONA LÓPEZ

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Dr. **CARLOS JOSÉ LÓPEZ HERRERA**, Científico Titular del Consejo Superior de Investigaciones Científicas adscrito al Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible (CSIC-Córdoba) y director de la presente Tesis Doctoral.

INFORMA:
Que D. **JUAN MANUEL ARJONA LÓPEZ**, ha realizado bajo mi dirección y supervisión, el trabajo experimental titulado "Control integrado de la podredumbre blanca del aguacate mediante métodos biológicos y químicos" donde se han completado con éxito todos los objetivos planteados en dicho trabajo de investigación.

Que dicha Tesis Doctoral se va a presentar como compendio de publicaciones, las cuales se indican a continuación.

Artículos en revistas SCI elaboradas durante el periodo de tesis doctoral y relacionada con el contenido de las tesis:

Arjona-Lopez, J.M., Telengech, P., Jamal, A., Hisano, S., Kondo, H., Dafny Yelin, M., Arjona-Girona, I., Kanematsu, S., Lopez-Herrera, C.J., and Suzuki, N. (2018). Novel, diverse RNA viruses from Mediterranean isolates of the phytopathogenic fungus, *Rosellinia necatrix*: insights into evolutionary biology of fungal viruses. *Environmental Microbiology*. 20: 1464-1483. doi:10.1111/1462-2920.14065.

Arjona-López, J.M., Tienda, S., Arjona-Girona, I., Cazorla, F.M., and López-Herrera, C.J. (2019) Combination of low concentrations of fluazinam and antagonistic rhizobacteria to control avocado white root rot. *Biological Control*. 136: 103996, doi: 10.1016/j.biocontrol.2019.05.015.

Arjona-López, J.M., Capote, N., and López-Herrera, C.J. (2019) Improved real-time PCR protocol for the accurate detection and quantification of *Rosellinia necatrix* in avocado orchards. *Plant and Soil*. 443: 605-612, doi: 10.1007/s11104-019-04215-6.

Otras aportaciones destacables que han surgido de la presente Tesis Doctoral son siete contribuciones a congresos:

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Arjona-López, J.M., Arjona-Girona, I., Ariza, M.T., Cretazzo, E., Velasco, L. y López-Herrera, C.J. Presencia de micovirus en aislados de *Rosellinia necatrix* y *Phytophthora cinnamomi*. XVIII Congreso de la Sociedad Española de Fitopatología, 20-23 de septiembre de 2016, Palencia, España. Póster 192.

Arjona-López, J.M., Suzuki N., López-Herrera, C.J. Detection and identification of new mycoviruses in *Rosellinia necatrix* isolates from avocado crops. XIX Congreso de la Sociedad Española de Fitopatología, 8-10 de octubre de 2018, Toledo, España. Oral 27.

Arjona-López, J.M., Arjona-Girona, I., López-Herrera, C.J. Efecto de Fluazinam sobre oomicetos y hongos patógenos y no patógenos habitantes en suelo del cultivo de aguacate: *Phytophthora cinnamomi*, *Rosellinia necatrix*, *Entoleuca* sp. y *Neofusicoccum* sp. XIX Congreso de la Sociedad Española de Fitopatología, 8-10 de octubre de 2018, Toledo, España. Póster 050.

Arjona-López, J.M., Tienda, S., Arjona-Girona, I., Cazorla, F.M. López-Herrera, C.J. Control integrado mediante la combinación de métodos biológico y químico para el control de la podredumbre blanca en aguacate. XIX Congreso de la Sociedad Española de Fitopatología, 8-10 de octubre de 2018, Toledo, España. Póster 051.

Arjona-López, J.M., López-Herrera, C.J. Caracterización y biocontrol de novedosos hongos antagonistas sobre la podredumbre blanca del aguacate. XIX Congreso de la Sociedad Española de Fitopatología, 8-10 de octubre de 2018, Toledo, España. Póster 052.

Arjona-López, J.M., Capote, N., Melero-Vara, J.M., López-Herrera, C.J. Valoración del efecto de fluazinam sobre el control de la podredumbre blanca del aguacate mediante detección y cuantificación molecular de *Roselinia necatrix* en suelo. XIX Congreso de la Sociedad Española de Fitopatología, 8-10 de octubre de 2018, Toledo, España. Póster 110.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 16 de diciembre de 2019.

Firma del director

Fdo.: CARLOS JOSÉ LÓPEZ HERRERA

“La tierra no es una herencia de nuestros padres, sino un préstamo de nuestros hijos”

Mahatma Gandhi

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Summary

Spain is the highest avocado producer country in the European Union. White root rot caused by the ascomycete *Rosellinia necatrix* is one of the most important diseases of this crop in the southern coastal area of Iberian Peninsula due to its difficult management. We studied different integrated control methods of this disease and its possible combinations. First, we detected and identified mycoviruses in *R. necatrix* isolates and their relation with the hypovirulence factor as a future virocontrol method. As well we obtained novel antagonistic fungi (*Entoleuca* sp. isolates) in which we analyzed mycoviruses presence, their growth under different temperatures, their vegetative compatibility groups and their biocontrol effect against the disease. We improved a qPCR technique for the detection and quantification of this pathogen from avocado orchards soil samples, thus we used this novel technique to assess the inoculum quantity of *R. necatrix* in the soil after the treatments of the fungicide fluazinam that we carried out in commercial orchards. Furthermore we performed an integrated control of this disease combining low concentrations of fluazinam with antagonist organisms such as rhizobacteria strains and/or non-pathogenic *R. necatrix* isolates. In this work we have studied all possible chemical and biological control methods against this disease nowadays, even some of them can be applied directly in field conditions.

Resumen

España es el mayor productor de aguacate en la Unión Europea. La podredumbre blanca radicular causada por el hongo ascomiceto *Rosellinia necatrix* Prill. es una de las enfermedades más importantes de este cultivo en la zona costera sur de la península Ibérica por su difícil manejo. Estudiamos diferentes métodos de control integrado de esta enfermedad y sus posibles combinaciones. Primero detectamos e identificamos micovirus infectando aislados de *R. necatrix* y su relación con el factor de hipovirulencia como un futuro método de virocontrol. También obtuvimos novedosos hongos antagonistas (aislados de *Entoleuca* sp.) en los que analizamos la presencia de micovirus, su crecimiento a diferentes temperaturas, sus grupos de compatibilidad vegetativa y su efecto de biocontrol contra la enfermedad. Mejoramos una técnica de qPCR para la detección y cuantificación de este patógeno en suelo a partir de muestras procedentes de fincas de aguacate, que fue utilizada como novedosa técnica para evaluar la cantidad de inóculo de *R. necatrix* presente en el suelo tras los tratamientos del fungicida fluazinam que realizamos en fincas comerciales para el control de la enfermedad. Además realizamos un control integrado de esta, combinando dosis bajas de fluazinam con organismos antagonistas como cepas de rizobacterias y/o aislados no patogénicos de *R. necatrix*. En este trabajo se han estudiado todos los posibles métodos de controles biológicos y químicos utilizables contra esta enfermedad hoy en día, pudiendo ser alguno de ellos de aplicación inmediata en campo.

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Chapter 1: Introduction and Objectives

Introduction

Taxonomy of avocado

Avocado belongs to *Lauraceae* family, genus *Persea*, subgenus *Persea* and specie *Persea americana* Mill. (Barrientos-Priego et al., 2008). *Persea* genus is divided in two subgenera: *Persea* and *Eriodhapne* (Kopp, 1966). *Persea* genus contains approximately 85 species distributed from the south of United States (*P. borbonia*) till Chile (*P. lingue*). Exceptionally, the species *P. indica* is distributed out of the American continent and located in Canary, Madeira and Azores islands.

Three avocado races are recognized: Mexican, Guatemalan and Antillana. Mexican race has the advantages of being cold resistant and high avocado oil content, Guatemalan race presents a thick shell, which gives a fruit resistance in transport, and race Antillana is adapted to tropical climate and, used as rootstock, is more tolerant to salinity (Fig. 1) (Barrientos-Priego et al., 2008).

Avocado trees are formed by two parts: tree top (aboveground) and roots (underground), the first is the cultivar grafted onto the rootstock, while the root comes from the rootstock.

Fig. 1. Possible centers of origin of avocado races (Storey et al., 1986).

The most cultivated variety in the World, and in Spain, is “Hass”. Coming from Guatemalan race, this variety was patented in California (USA) in 1935, and its fruits have rugged skin, with a creamy pulp and excellent flavor. Other varieties such as “Fuerte” and “Bacon” are hybrids between Mexican and Guatemalan races. “Reed” variety has round fruit with green color. The variety “Pinkerton” probably comes from other two varieties, such as “Hass” and “Rincon”, its fruit being dark green with rugged skin.

Avocado history

The origin of avocado word (Etymology) is “ahuakatl” (with a secondary meaning "testicle" probably based on its resemblance) coming from Nahuatl (aztecan language) derived to the Spanish word “aguacate” (Online Etymology Dictionary, 2019). Its fruit is known by different names depending on the language and country, thus it is known as “aguacate” in Spanish speaking countries, excepting in some South American countries such as Argentina, Bolivia, Chile, Peru and Uruguay where it is named as “palta,” and “cura” in Venezuela. In English speaking countries is known as “avocado,” whereas “abacate” is used in Portuguese (Knight, 2007).

Avocado is originally from Mexico, with the first records of this crop dating back to 1500 B.C. and its origin is estimated in south central Mexico and northern Guatemala between 7000 and 5000 B.C. (Popenoe, 1920; Smith, 1969; Storey et al., 1986). The first known written description about avocado fruit is the book “Suma de Geografía” written by Martin Fernández de Enciso in 1519. The avocado tree was disseminated to the rest of the American continent and Spain by Spanish conquers and today this plant is cultivated in all continents, except the Antarctica.

Importance of avocado cultivation

The avocado fat is the most important component from the dry substance of mesocarp, and is responsible for the flavor and texture. Its consumption has several benefits to the human healthy, i.e., reduction of cardiovascular diseases, due to its α-tocopherol content; prevention of colon, breast and prostate cancer, due to its content of β-sitosterol, which inhibits the intestinal absorption of cholesterol, thus reducing the levels of total cholesterol and low-density lipoprotein (LDL); and decreasing the risk of eye disorders and cataracts, due to its content of lutein. In addition, it has been observed a beneficial effect of avocado consumption in human patients’ asthma and rheumatoid arthritis (Pérez Rosales et al., 2005).

Leaves can be used as expectorants, fruit and seed are used to produce natural medicine to remove microbes and parasites, against dysentery and some digestive disorders. The protein energy of the fruit combined with the vitamins and mineral salts give aphrodisiac properties (Avocado uses, 2019), even the avocado seed can be used as fuel bioenergy for domestic or industrial heating (Perea-Moreno et al., 2016).

Avocado contains monounsaturated and polyunsaturated fats which cause less damage to liver than saturated fats, being a healthy substitute of other common fats used (Table 1).

Table 1. Nutritional comparison of avocado with other common fats (California Avocado Commission, 2019).

	Avocado	**Butter**	**Sour cream**	**Margarine**	**Cheddar cheese**	**Mayonnaise**
Serving size	50 g	1 [a]TBPS	2 TBPS	1 TBPS	1 [b]Oz	1 TBPS
Calories	80	100	45	100	110	90
Total fat (g)	8	12	4.5	11	9	10
Saturated fat (g)	1	7	3	2	5	1.5
Cholesterol (mg)	0	30	10	0	30	5
Sodium (mg)	0	90	10	95	180	90

[a] 1 TBPS (Tablespoon) = 15 g

[b] 1 Oz = 28.35 g

Avocado is rich in healthy nutrients, it provides more requirements of diary nutrients respective to less calories wasted and does not contain cholesterol (Table 2).

Table 2. Nutritive value averaged per each 50 g of avocado serving (California Avocado Commission, 2019).

Calories	**Fatty acids (%)**	**Minerals (mg/% [a]DV)**	**Vitamins (mg/%DV)**	**Protein (g)**
80	Saturated fat 6 – 22	Calcium 10/0	C 4/4	1
	Monounsaturated fat 66 – 72	Iron 0.3/2	E 1/6	
	Polyunsaturated 8 – 11	Potassium 250/6	K 11/10	
		Phosphorus 30/2	B6 0.1/6	
		Magnesium 15/4	Thiamin 0.04/4	
		Zinc 0.3/2	Riboflavin 0.1/8	
		Copper 0.1/10	Niacin 1/6	
		Manganese 0.1/4	Folate 45/10	
			Pantothenic acid 0.7/14	

[a] DV: Daily Value (DV) is based on a caloric intake of 2,000 calories for adults and children four or more years of age

Avocado cultivation

The production and distribution of avocado industry began in XX century specifically located in California (Díaz Robledo, 1997). Nowadays avocado is cultivated in nearly 70 countries, the main producer countries are located in the American continent (Fig. 2). The total production in the world during 2017 was 5,924,398 tons, from an acreage of 587,278 ha (FAOSTAT, 2019).

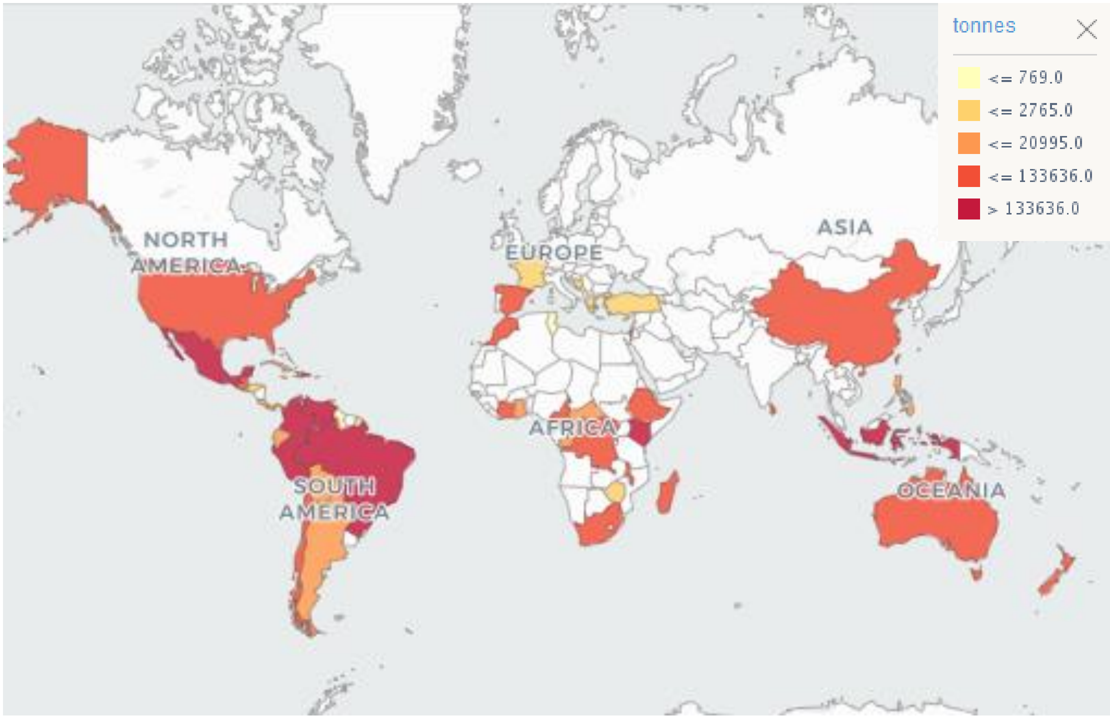

Fig. 2. Distribution of avocado production in the world during 2017 (FAOSTAT, 2019).

Mexico is the largest avocado producer country in the world during 2017, with a production higher than two million tons, obtaining a constant production during all year and exporting mainly to USA, Europe and Japan. Spain is within the 20 top avocado producing countries in the world, having the 16th position of this rank during 2017 and is the largest producer country in the European Union (Table 3) (FAOSTAT, 2019).

Production in Spain

Number of avocado orchards and production levels have progressively increased since this crop was established in Spain around the 1960´s, displaying a production of 92,936 tons covering a harvested area of 11,812 ha during 2017 (Fig. 3) (FAOSTAT, 2019). Around 80% of this production is exported to Europe mainly to France.

Avocado is mainly cultivated in the southern coast of Andalusia where it is alternative to traditional rainfed crops such as olive, almond and grapes, and Canary Islands. Malaga and Granada provinces are the highest producers in the south of the Iberian Peninsula with 44,863 and 30,085 tons in an area of 6,804 ha and 2,584 ha respectively (Table 4) (MAPAMA, 2019).

Table 3. Avocado production values in the main 20 producer countries during 2017 (FAOSTAT, 2019).

Ranking	Country	Production (tons)
1	Mexico	2,029,886
2	Dominican Republic	637,688
3	Peru	466,758
4	Indonesia	363,157
5	Colombia	314,275
6	Brazil	213,041
7	Kenya	194,279
8	Venezuela	133,922
9	Chile	133,636
10	United States of America	132,730
11	Guatemala	125,596
12	China	124,110
13	Israel	110,000
14	Haiti	97,520
15	Malawi	97,358
16	**Spain**	**92,936**
17	Cameroon	71,235
18	Democratic Republic of the Congo	65,558
19	South Africa	62,840
20	Ethiopia	57,120

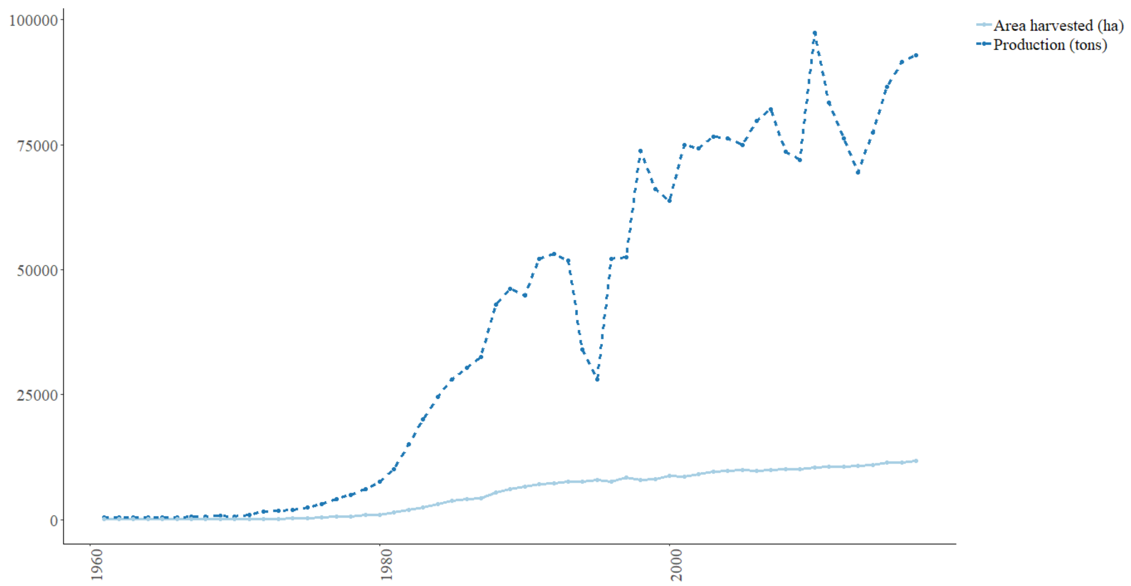

Fig. 3. Evolution of avocado production (tons) and area harvested (ha) in Spain since 1961 till 2017 (FAOSTAT, 2019).

Table 4. Harvested area and production of avocado in Spain distributed by provinces and regions (MAPAMA, 2019).

Provinces	Regions	Total area (ha)	Harvested area (ha)	Production (tons)	Yield (tons/ha)
Malaga		6,804	6,232	44,863	7.20
Granada		2,584	2,583	30,083	11.65
Cadiz		346	309	4,326	14.00
Huelva		25	25	438	17.52
Almeria		4	4	48	12.00
ANDALUCIA		**9,763**	**9,153**	**79,758**	**8.71**
Tenerife		1,429	1,275	9,101	7.14
Las Palmas		178	176	1,233	7.01
CANARIAS		**1,607**	**1,451**	**10,334**	**7.12**
Alicante		189	149	1,341	9.00
Valencia		206	82	615	7.50
Castellon		18	4	60	15.00
C. VALENCIANA		**413**	**235**	**2,016**	**8.58**
BALEARES		**8**	**8**	**94**	**11.75**
Tarragona		7	7	42	6.00
CATALUÑA		**7**	**7**	**42**	**6.00**
R. DE MURCIA		**14**	**2**	**14**	**7.00**
TOTAL SPAIN		**11,812**	**10,856**	**92,258**	**8.50**

The avocado price paid to farmers has increased progressively along the last ten years, achieving an average of 214 € per 100 kg in 2017. This could be the main reason of the raising avocado orchards in Spain nowadays, because its crop profitability for farmers (Table 5).

Importance of crop diseases

Plant diseases are an important limiting factor of agricultural production in the world, pathogens (fungi, chromista and bacteria) can cause crop losses of 21% (Oerke, 2006). Even, they can cause great epidemics, such as the Great Famine in Ireland (1845 – 1849) with a potato blight produced by the oomycete *Phytophthora infestans*, inducing a decreasing of Ireland population between 20 – 25% because of the people deaths (around one million) and emigration (about 1 million) during this period (Ó Gráda, 2006).

The study of these problems has been known since ancient times, i.e., Theophrastus (371 – 287 B.C.) but it was scientifically improved in the Early Contemporary period, with the appearance of the microscope and it developed later in the 19th century (Ainsworth, 1981). thus, the control strategies to reduce the production losses caused by the pathogens, using sustainable practices, are really important nowadays (Mehta, 2014).

Table 5. Evolution of avocado production (tons), price prearranged for farmers (€/100 kg) and value (thousand €) during ten years (2007-2017) in Spain (MAPAMA, 2019).

Years	**Production (tons)**	**Average of price prearranged for farmers (€/100 kg)**
2007	82,116	113.83
2008	73,585	121.48
2009	71,931	120.80
2010	75,655	131.55
2011	98,535	131.22
2012	76,337	125.76
2013	69,427	143.48
2014	79,886	146.94
2015	86,636	157.90
2016	91,530	211.67
2017	92,936	214.62

Main avocado diseases

The most important and destructive disease of avocado orchards worldwide is avocado root rot, caused by the oomycete *Phytophthora cinnamomi* Rands. (Zentmyer, 1980; Pegg et al., 2002). This pathogen was first detected in 1922 on cinnamon trees in Sumatra and later on avocado crops from the highest producer countries such as Mexico, Brazil (Zentmyer, 1977) or USA (California) (Zentmyer, 1985). This pathogen have been found in over 70 countries all over the world affecting around 1,000 plants (Zentmyer, 1985).

Other important diseases of avocado depending on its incidence in different countries are the following: white root rot caused by *Rosellinia necatrix* Prill. (Sztejnberg et al., 1987; López-Herrera, 1989), *Armillaria* root rot (*Armillaria mellea* (Vahl) P. Kumm. and *A. tabescens* (Scop.) Emel) (Ohr and Zentmyer, 1994), Sunblotch induced by viroid ASBV (López-Herrera et al., 1987), *Verticilium* wilt (*Verticillium dahliae* Kleb.) (Zentmyer, 1994), one of the most important foliar diseases is induced by *Cercospora purpurea* Cooke (Stevens, 1922) and the most common fruit diseases are caused by *Colletotrichum gloeosporioides* Penz. and Sacc. (Pegg et al., 2002), *Dothiorella* spp., *Lasiodiplodia theobromae* Griffon and Maubl., and *Phomopsis perseae* Zerova (Anderson, 2003).

In Spain, the main fungal diseases of avocado orchards are avocado root rot, avocado white root rot (López-Herrera, 1989) and dieback produced by fungi belonging to *Botryosphaeriaceae* family which incidence has been progressively increased in this avocado producing area becoming a new disease problem recently (Arjona-Girona et al., 2019).

Since 1986, the disease incidences in avocado orchards caused by the first two pathogens have progressively increased in the provinces of Malaga and Granada reaching 39% for *R. necatrix* and 35% for *P. cinnamomi*, and 7% to orchards affected by both pathogens (Pérez Jiménez et al., 2003c) (Fig. 4 and 5). Furthermore, *R. necatrix* is difficult to control and scant effective control methods have been developed under field conditions (ten Hoopen and Krauss, 2006), while *P. cinnamomi* is easier to manage, because several commercial tolerant avocado rootstocks such as "Duke-7", "Thomas" or "Toro Canyon" were developed (Coffey, 1987), and the chemical control is highly efficient using systemic fungicides such as Metalaxyl formulated as 25% EC, which can be applied by irrigation system, and the other chemicals such as Fosetyl-Al can be applied by foliar spray or injected into the trunk of avocado tree (Coffey, 1987). In Canary Islands avocado root rot is the most important disease of this crop (Hernández-Hernández et al., 1999) and branch avocado dieback has been recently detected, its incidence being increasing lately (Siverio et al., 2018).

Fig. 4. Aerial view of damages evolution caused by *Phytophthora cinnamomi* in an avocado orchard located in Malaga (Spain) (Google Earth Pro, 2002, 2018).

White root rot disease

Rosellinia necatrix Prill. (anamorph: *Dematophora necatrix* Harting) is a soil-borne phytopathogenic ascomycete, which is the causal agent of white root rot, affecting about 170 plant species and 63 genera (ten Hoopen and Krauss, 2006). First, De Notaris, (1844) named the genus *Rosellinia*, because the stromatic character of its fructification, its taxonomic position within the pyrenomycetes has not always been clear. The asexual phase of this fungus was scientifically identified as *Dematophora necatrix* (Hartig, 1883), previously known as *Rhizomorpha necatrix*.

Next, *D. necatrix* was included in the genus *Rosellinia* by Berlese, (1982) because of the ascocarps morphological descriptions of the fungus *Rosellinia aquila* De Not. Other authors confirmed this taxonomic classification when they observed the perithecia on fruit tree roots infected by this pathogen (Sztejnberg et al., 1980; Nakamura et al., 2000; Pérez - Jiménez et al., 2003a). Thus, the teleomorph was named as *R. necatrix*, which is classified on the phylum Ascomycota, subphylum Pezizomycotina, class Sordariomycetes, subclass Xylariomycetidae, order Xylariales and family Xylariaceae (Prillieux, E., 1904). In Europe, this pathogen was first detected by Viala (1886) infecting grapevine crop.

Fig. 5. Aerial view of an avocado orchard with an evolution of damages caused by *Rosellinia necatrix* in Malaga (Spain) (Google Earth Pro, 2007, 2015).

Growth and survival of *R. necatrix* needs high levels of oxygen, humidity and cellulose sources in the soil. Furthermore, this fungus increases saprophytic and parasitic activities with high levels of organic matter, which helps to spread it in the soil (Araki, 1967).

On potato dextrose agar growth medium, the young mycelium of *R. necatrix* is white and cottony, changing to dark color later. The most important microscopic characteristic of this fungus is the presence of pear-shaped swellings in the hypha, located close to the septum, which has been widely used to identify the species. These pyriform swellings are larger in superficial mycelium than in submerged mycelia (Khan, 1959). The optimal growth temperature for this pathogen ranges between 22 – 25 °C in darkness (García-Jiménez et al., 2004; Ruano-Rosa, 2006; Dafny-Yelin et al., 2018).

This pathogen produces three different types of spores (chlamidospores, conidiospores and ascospores), but it is unclear the role of them in the epidemiology of the disease, because the pathogen is spread directly through the root contacts among host plants (Pérez-Jiménez et al., 2003a).

The asexual life cycle of *R. necatrix* (Fig. 6) is developed with chlamidospores and conidiospores. The first are spherical and are generated by condensation of cell wall pyriform swellings, rarely being found under natural and artificial conditions (Makambila, 1976), and the conidiospores are originated at the end of conidiogenous cells in the sclerotia or brown mycelial masses. The sclerotia of *R. necatrix* are spherical with black nodules, which are located on invaded roots and connected from their base with the subcortical mycelium (Viala, 1886).

The sexual spores of *R. necatrix*, called ascospores, are generated inside of perithecia and are expelled outside within a mucilaginous mass through their papillate ostiole when perithecia are ripening (Fig. 6). The released ascospores germinate over their ventral cleft producing a mycelium with pyriform swellings, which infects the feeder roots of the tree again therefore initiating the asexual cycle of this fungus. The perithecia production needs a long period with high environmental humidity both under natural and *in vitro* conditions (Nakamura et al., 2000; Pérez-Jiménez et al., 2003a). These structures have been found on apple trees (Teixeira de Sousa, 1991) aas well as on avocado roots (Pérez-Jiménez et al., 2003a).

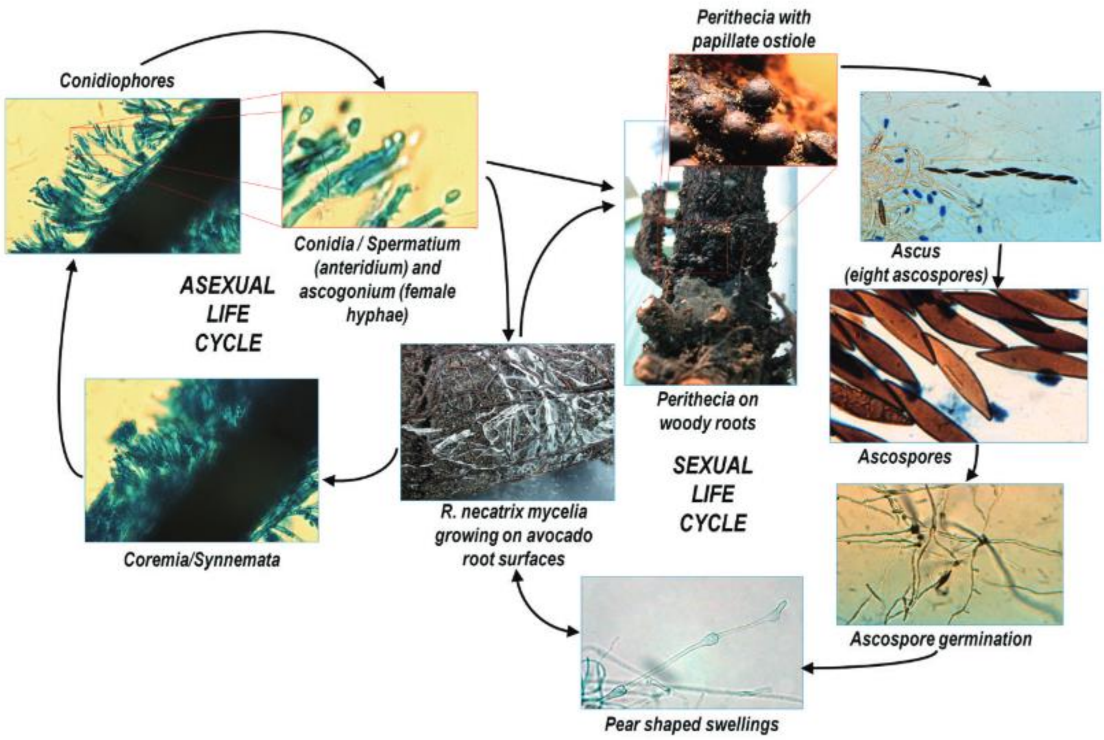

Fig. 6. Life cycle of *Rosellinia necatrix*. Sexual and asexual life cycle are exposed with their respective reproductive structures (Pliego et al., 2012).

The infective initial and dispersal process of white root rot disease occurs underground, having a high saprophytic behavior, mostly on dead wood. The mycelium colonizes the roots of host plants, producing hyphal aggregates which penetrate in woody roots and cause disease on plants, showing aerial symptoms when the infection process is advanced (Delatour and Guillaumin, 1985).

The symptoms on the roots can be observed on their surfaces with white cottony mycelium which penetrates in the root tissue. The pathogen is located between the bark and the wood showing typical fan-like mycelia which colonize the root system and take a dark color and cause the disease (Fig. 7). The main fungal dispersion is by roots contacts from diseased trees to healthy trees, the infection process begins at different points and depths in the soil. Trees infected by this pathogen do not always show aerial symptoms, making its detection a difficult process.

Fig. 7. Avocado root colonized and affected and colonized by *Rosellina necatrix* showing typical fan-like white mycelium. Picture taken by C.J. López-Herrera.

The aboveground symptoms on leaves and stem can occur either in a short period of time, because the infected trees decline its vigor, showing wilted and dried leaves, and finally these trees die, or slowly, when the growth of infected trees is delayed and the foliage can be reduced in these trees, eventually appearing chlorotic and wilting leaves, with twigs, branches and leaves death. The speed of aboveground symptoms depends on environmental and soil factors such as temperature or humidity, tree health and pathogen virulence, increasing every year when the temperature and humidity are suitable and the trees die (Fig. 8) (Guillaumin et al., 1982).

White root rot detection

The detection of this disease is difficult, it has commonly been carried out by conventional phytopathological techniques to isolate the fungus from infected roots or using bait tree twigs

(Eguchi et al., 2009), but both methods are time-consuming and take several days to obtain clear results (Fig. 9).

During last decades, molecular tools (conventional PCR and qPCR) have been successfully developed to detect *R. necatrix* in natural and artificial infested soils (Schena et al., 2002; Schena and Ippolito, 2003; Ruano-Rosa et al., 2007). In addition, the detection and quantification of this pathogen have been carried out successfully in artificially infested soil (Shishido et al., 2012; Pasini et al., 2016).

Fig. 8. Avocado trees with typical symptoms of white root rot disease located in "La Mayora" orchard (Malaga, Spain). Picture taken by J.M. Arjona-López.

Fig. 9. Avocado roots affected by *Rosellinia necatrix*. Picture taken by C.J. López-Herrera.

White root rot development

The infection process of *R. necatrix* in young mulberry tree roots has been described by boring and dissolving cork cell and, on rare occasions, by wedging them. However, the invasion of adult roots into the inner tissues seems to take place mainly through the suberized closing layers of the lenticels, generally as hyphal strands (Sakurai, 1952). Furthermore, the penetration of *R. necatrix* in apple tree roots has been known to occur in different phases and forms of hyphal aggregates (Tourvieille de Labrouhe, 1982).

The infection process on avocado tree roots described by Pliego et al. (2009) using a high virulent *R. necatrix* isolate marked with fluorescence and inoculated on 6 month-old plants was similar as described in the references given in the previous paragrah. The visualization of light fluorescence described the penetration on avocado roots at the crown region, through the lenticels and junctions between epidermal cells. This imaging of infection process reveals that primary infection on roots through the junctions between epidermal root cells occurs simultaneously at several random positions along the root axis.

R. necatrix distribution

R. necatrix has a worldwide distribution in different hosts and was detected in all continents except Antarctica. This fungus has been reported on Japanese pear, apple and grapevine orchards in Japan (Arai, 1989; Kanadani et al., 1998; Arakawa et al., 2002) and on cherry trees in Iran (Behdad, 1975) where distribution was widespread. In addition, *R. necatrix* has been detected in other important avocado producing countries such as Mexico, Dominican Republic, Colombia, Brazil, USA (California), China, Democratic Republic of Congo and Ethiopia (CABI, 2019). Furthermore, this soil-borne pathogen has been found infecting avocado trees in Israel (Sztejnberg et al., 1987) and South Africa (van den Berg et al., 2018) (Fig. 10).

Control of white root rot disease

The control of this disease is difficult because this pathogen can tolerate dry environments and acidic soils, it has a wide range of hosts, can survive deeply into the soil and it is tolerant to many common fungicides (Khan, 1959). Treatments against the disease must be carried out for a long period response in two ways, pre-planting and post-planting; in this last case, none of treatments might affect the crop. The main methods to reduce the disease are cultural practices, genetic resistance and physical, chemical, biological and integrated controls.

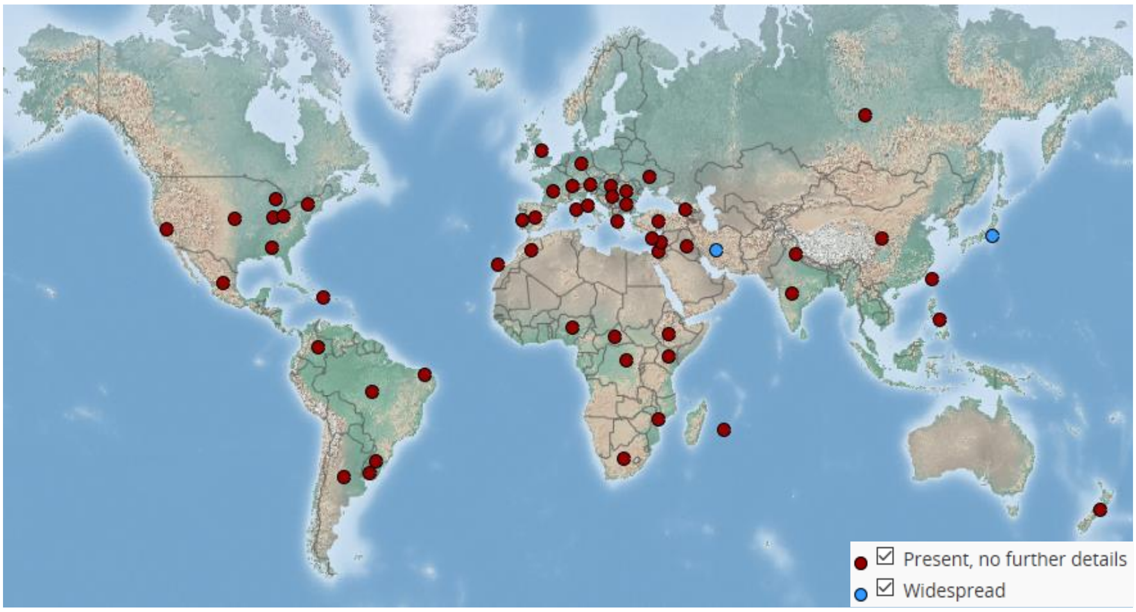

Fig. 10. World distribution by countries of *Rosellinia necatrix* (CABI, 2019).

Cultural practices

The cultural practices to reduce the soil inoculum incidence in an infested established orchard are focused on destruction by burning affected plants. All organic matter from roots should be removed and burned to avoid the dispersion of any organic material to other non-infested soil, because *R. necatrix* can colonize other organic matter (Mendoza García et al., 2003). In addition, the seeds used for a subsequent germination and plant growth must be previously treated with hot water (53 – 55 °C for 25 – 30 min) (Garcia-Jiménez et al., 2004). At the same time, avoiding the fungal infection on nursery plants is required, being necessary to check periodically the pathogen presence in their roots. Furthermore, the water irrigation management can induce tolerance on avocado plants as reported by Martínez-Ferri et al. (2019) demonstrating that mild water stress can induce a cross factor priming which increase avocado tolerance to *R. necatrix* of a susceptible rootstock.

Commonly, *R. necatrix* has been detected by conventional techniques of root isolation which is time-consuming and non-accurate method because other soil fungi can growth faster than this pathogen. Nevertheless, novel molecular detection methods based on qPCR are highly reliable and quicker to prevent the occurrence of *R. necatrix* before orchards planting. Besides valuable information on the evolution of the inoculum level of the fungus in the soil after physical, chemical or biological control treatments are obtained (Schena et al., 2002; Schena and Ippolito, 2003; Ruano-Rosa et al., 2007).

Physical control

The main physical method used to control this fungus is soil solarization. This technique arose in Israel during the late 1970s and consists of a hydrothermal process achieved by covering wet soil with plastic transparent sheet to absorb solar radiation. The temperature can raise to 45 – 50 °C in the surface layer of soil and 40 – 45 °C at 25 cm depth, affecting the soil pathogens by its exposure to high temperatures. This method has been assayed successfully against *R. necatrix* in established orchards, reporting 75% mortality of *R. necartix* at 60 cm depth after 56 days of solarization for apple trees in Israel (Sztejnberg et al., 1987) and complete inoculum destruction at the same depth after six weeks of solarization on avocado orchards in southern Spain (López-Herrera et al., 1998, 1999). Additionally, if the pathogen has a weakened infestation in the soil and infection on roots it could be more prone to antagonistic effects, due fungi such as *Trichoderma* spp. that can colonize solarized soils easier than the pathogen (Katan, 1987).

Genetic resistance

Unusually, few research efforts have reported genetic resistance in different crops affected by *R. necatix* (ten Hoopen and Krauss, 2006). In the same way, there is no many studies about the tolerance of avocado rootstock to soil diseases, including the genetic resistance of this crop to this soil-borne pathogen. Commercial avocado rootstocks tolerant to *P. cinnamomi* such as "Duke-7", "Thomas" or "Toro Canyon" have been checked for susceptibility to *R. necatrix* (Pérez Jiménez et al., 2003b). Up to date, Barceló-Muñoz et al. (2007) reported some tolerant avocado rootstock candidates which still are not available to growers. Currently, this research staff is developing commercial avocado rootstock tolerant to *R. necatrix* by testing the selected candidates in commercial avocado orchards affected by white root rot.

Biological control

Traditionally, growers use chemical compounds to reduce the disease incidence in crops. Conversely, new environmental concerns, chemical safety of growers and the emergence of different resistance mechanisms from pathogen are reducing the use of pesticides and their applications (de Waard et al., 1993; Gullino and Kuijpers, 1994; Harman and Kubicek, 1998). In this way, an alternative to the use of chemicals is the application of biological control agents (BCA) applied alone or combined with low concentration of chemical compounds to reduce this effect and suitable to be used as an integrated disease control. Different BCA's have been reported as effective tools against soil-borne plant pathogens (Papavizas, 1973; Deacon, 1991; Harman, 2000).

Antagonistic fungi

Several fungal species have been successfully used as antagonistic biological control agents, which have reduced the mycelial growth *in vitro* as the entomopathogen *Beauveria bassiana* (Reisenzein and Tiefenbrunner, 1997) and *Clonostachys* spp., *Fusarium* sp., *Trichoderma harzianum, Trichoderma virens* and *Trichoderma* sp. were reported to decrease disease symptoms caused by *R. necatrix* in different crops, such as cocoa seedlings (Mendoza García et al., 2003); as well as *Glomus heterosporum* and *Glomus* sp. on apple seedlings (Bhardwaj et al., 2000), or *Sordaria* spp. on black pine, cucumber and spinach as host plants (Watanabe, 1991). One of the most common antagonistic fungi, *Thrichoderma* spp., have been successfully used as BCA against *R. necatrix* on apple orchard (Sztejnberg et al., 1987) and avocado plants (Ruano-Rosa and López-Herrera, 2009; Ruano-Rosa et al., 2010). A recent study has described *Entoleuca*, novel Xylariaceae fungi isolated from the avocado rhizosphere, as a new potential BCA against this pathogen on avocado plants (Arjona-Girona and López-Herrera, 2018). Conversely, the species *Entoleuca mammata* has been described as the causal agent of Hypoxylon canker in forest trees such as poplars (Anderson, 1964). Furthermore, non-pathogenic *R. necatrix* isolates have shown a successful control against *R. necatrix* pathogenic isolates (Ruano-Rosa and López-Herrera, 2006).

Mycoviruses

Mycoviruses have been proposed as another possible biocontrol tool of fungal pathogens, because these organisms could confer hypovirulence factor, in this way reducing the virulence of their host fungus, thus being another type of BCA to control the disease caused by *R. necatrix* (Matsumoto et al., 2002).

Many studies have reported different rates of virus-infected fungi which varied between low percentages to more than 90% (Hillman et al., 2018). In the case of *R. necatrix*, two previous studies reported a positive rate of viruses infection nearly of 20% in a total of 298 Japanese and Australian isolates (Arakawa et al., 2002) and in a total of 424 Japanese isolates (Ikeda et al., 2004).

Traditionally mycoviruses transmission have been described as effective between compatible vegetative fungal strains. However, there are other recent techniques which have allowed mycoviruses transmission between incompatible isolates such as transfection system of purified virions to protoplasts generated from the virus-free isolates. The methodology followed for this technique is similar than DNA transformation, mixing fungal protoplast with purified virions, which was effective on *Cryphonectria parasitica* (Hillman et al., 2004) (Fig. 11A). Another transfer method is via protoplast fusion between incompatible strains. van Diepeningen et al. (1998) successfully transferred virus between different *Aspergilli* species. Additionally, this technique was successfully used when transferring mycoviruses between different genera of fungi, from *Fusarium poae* into *Aspegillus* strains (van Diepeningen et al., 2000) and *F. boothi* into another *Fusarium* species and *Cryphonectria parasitica* (Lee et al., 2011) (Fig. 11B). On the contrary, this last method is restricted to laboratory conditions because protoplast fusion involves the mixture of all cellular genetic components (nuclei and mitochondria) (Kondo et al., 2013). Additionally, Ikeda et al. (2013) reported other methodology of horizontal transmission between incompatible *R. necatrix* isolates after screening around 100 chemical compounds. They successfully achieved transmission with zinc compounds ($ZnCl_2$ and $ZnSO_4$), which attenuated the heterogenic incompatibility and allowed the mycoviruses transmission through anastomosis. This technique does not require fungal protoplast preparation and it can occur under natural conditions inside the same fungal species (Fig. 11C).

Therefore, one study carried out by Kanematsu et al., (2004) described horizontal transmission between compatible Japanese *R. necatrix* isolates with a Reovirus conferring hypovirulence to a virulent virus-free Japanese *R. necatrix* isolate tested on apple plants. In the same work, the authors cured the hypovirulent isolate of this Reovirus inducing the virulence of this strain on apple plants. Another work described the transfection of a Megabirnaviridae member named as Rosellinia necatrix megabirnavirus 1 between incompatible strains using purified virions conferring hypovirulence to *R. necatrix* Japanese isolate on apple plants (Chiba et al., 2009). These studies indicate that both mycoviruses could be potential BCAs on virocontrol. This is a novel biocontrol method which uses infective viruses against pathogenic organisms in important crops. Virocontrol was successfully carried out against the chestnut blight in field conditions transferring one hypovirus (Nuss, 1992), this disease is caused by the air-borne fungus *C. parasitica*. On the contrary, *R. necatrix* is a soil-borne fungus which rarely sporulate or its conidia germination rate is lower than 4%**.** Virocontrol against this soil-borne pathogen must be carried out with the introduction of a mycovirus with hypovirulence factor into a *R. necatrix* strain isolated from the target orchard, before placing this hypovirulent isolate into the soil. Then the new virus-carrying fungal strain can spread the virocontrol agent (mycovirus) as a donor after the introduction in an orchard (Kondo et al., 2013) (Fig. 12).

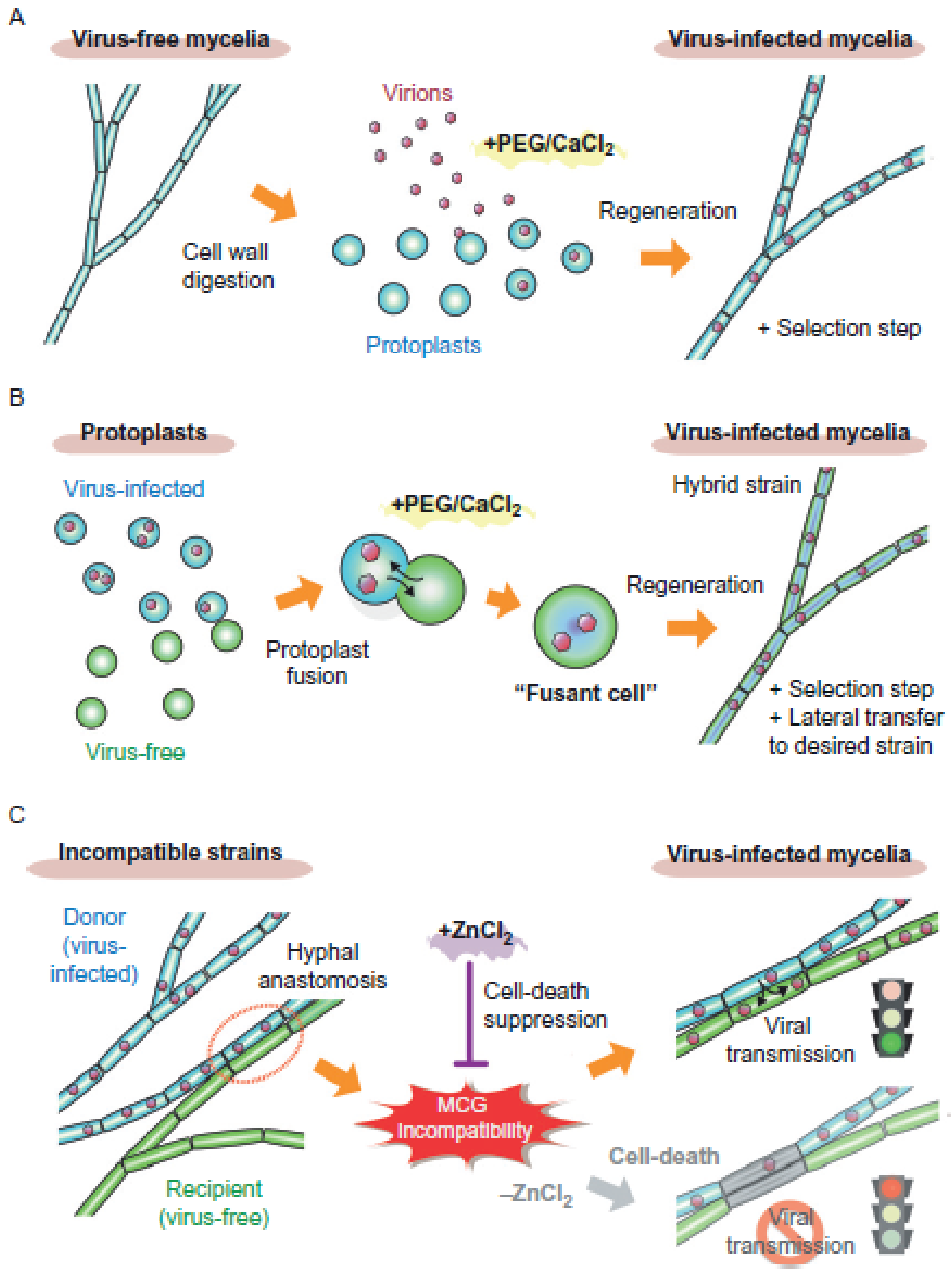

Fig. 11. Different techniques for mycoviruses transmission, virion transfection (A), protoplast fusion (B) and hyphal anastomosis with zinc compounds between incompatible strains (C) (Kondo et al., 2013).

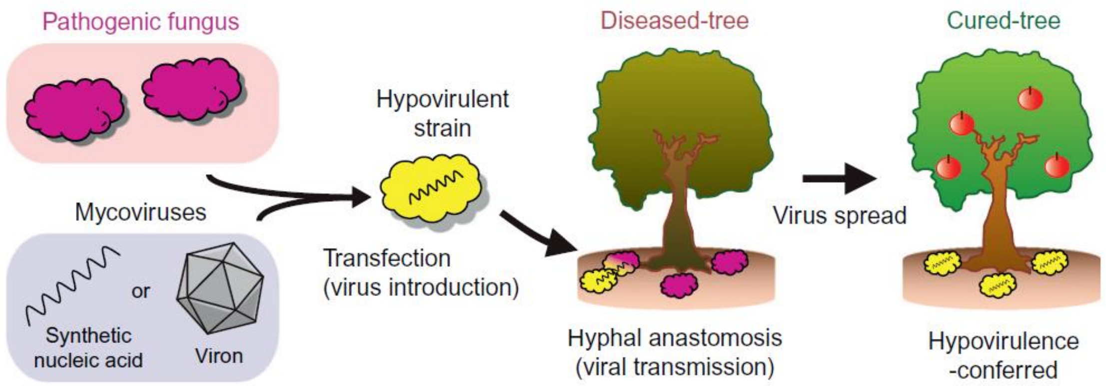

Fig. 12. Scheme of virocontrol methodology in field conditions and the expected virus to spread via hyphal anastomosis (Kondo et al., 2013).

Antagonistic bacteria

Two rhizobacteria species isolated from apple and peach, i.e., *Agrobacterium* spp. and *Pseudomonas* spp., have been assayed as effective BCAs with against *R. necatrix in vitro* (Yasuda and Katoh, 1989). Furthermore, *Bacillus* spp. (Sharma and Sharma, 2002) and the rhizobacterium species *Pantoea agglomerans* (Valdebenito-Sanhueza, 2001) have been successfully used against white root rot on apple plants. In recent studies, isolates of two rhizobacteria species, *Bacillus subtillis* (Cazorla et al., 2007) and *Pseudomonas chlororaphis* (Cazorla et al., 2006), from the rhizosphere of Spanish avocado trees have a potential antagonist activity against this disease on avocado plants producing antifungal compounds, hydrolytic enzymes, or volatile compounds (González-Sánchez et al., 2010; Calderón et al., 2013).

Organic materials

Organic materials such as Violacein from *Janthinobacterium lividum* (Shirata, 2000); culture filtrate of *Bacillus amyloloquefaciens* (Yoshida et al., 2001); and *Datura suaveolens* extract (Orozco Villareal, 1996) have been used as compounds to reduce the effect of white root rot disease.

Insects

Shiraishi et al. (1993) have reported the use of the insect *Mycophagous collembola* as an effective BCA against *R. necatrix.*

Chemical control

Several chemical compounds have been tested to control *R. necatrix*. The chemical control of avocado white root rot has been studied since 1915 using ferrous sulphate, mercury compounds, formaldehyde and carbon disulphide (Fawcett, 1915; Saccas, 1956; Behdad, 1976). The use of benzimidazole fungicides, thiophanate methyl and benomyl at 0.2 µg ml^{-1} a. i. inhibited completely or almost completely *R. necatrix* growth (Behdad, 1976). However, Shukla et al., (1973) reported an ED_{60} of 35 mg ml^{-1} for quintozene and 1585 mg ml^{-1} for Captan *in vitro* conditions. In addition, other studies reported the effect of several antibiotics on *R. necatrix* growth *in vitro* but only aureofungin inhibited completely the growth at the highest concentration used (40 µg ml^{-1}) (Sharma and Agarwala, 1967).

The use of isoprothiolane, a phosphorothiolate systemic fungicide/plant growth promoter, is recommended to control *R. necatrix* by the British Crop Protection Council, (2002). Treatments based on fumigants as metham-sodium and formaldehyde were most effective controlling fungal growth than other fungicides used on treated soil. Even formaldehyde did not reduce *Trichoderma* growth after its application, because soils treated with this chemical compound were quickly colonized by *Trichoderma*, which is a genus with a significant biocontrol potential (Mantell and Wheeler, 1973). Preplanting incorporation of 100 g m^{-2} of Dazomet (Basamid) in the first 50 cm of soil layer, followed by sprinkling water irrigation (1000 l m^{-3}) and covering the soil with a film of PVC during one month was useful as a commercial soil fumigant against *R. necatrix*. The soil could be cultivated three months after film removal when all chemical residues had been released (Nitta et al., 2002) Thus, effective control for periods up to two years were obtained (Nitta et al., 2002). Gupta, (1977) carried out field applications with carbendazim by soil drench on apple trees using a 0.1% suspension through holes 15 – 25 cm deep and trees recovery was shown after this treatment. This soil drench technique was also effective on mulberry trees in Japan using tridemorph (Mappes and Hiepko, 1984). Furthermore Sztejnberg et al. (1983) efficiently controlled white root rot for a period of at least nine months on an infested avocado orchard using methyl bromide by soil deep injection (up to 90 cm) with cold gas at 1500 kg ha^{-1} and by surface application with hot gas at 1000 kg ha^{-1}.

Fluazinam

Japanese producers of greenhouse grapevine and pear have successfully used thiophanate-methyl against *R. necatrix*, but they have changed by soil drench with the contact fungicide fluazinam which belongs to the pyridine group (Kanadani et al., 1998; Nitta et al., 1998), in spite of its higher price because it remains stable in the soil for longer than thiophanate-metyl, therefore reducing the number of chemical applications (ten Hoopen and Krauss, 2006). This contact fungicide is supplied by the Japanese company Ishihara Sangyo Kaisha, Ltd. (ISK Biosciences) and recommends its use to control late blight, tuber blight, Sclerotinia rot, scab and powdery scab on potatoes; gray mold and downy mildew on grapevines; scab and Alternaria blotch on apples; gray mold, melanoses (Diaporthe spp.) and mites on citrus; Sclerotinia rot and southern blight on peanuts; clubroot on crucifers; as well as white and violet root rots on fruit trees (ISK Biosciences, 2019).

Effective treatments against soil-borne disease are require delivery of fungicides to the target zone. Previous studies reported the movement and persistence of two benzimidazole fungicides and one piperazine applied to soil surface of the apple orchard, showing a movement to soil depth of only 6 cm (Sharma and Gupta, 1985). To avoid this restriction, Sugimoto, (2002) in Japan and Stephens et al., (2003) in Australia achieved great success on controlling white root rot in commercial apple orchards improving the efficacy of soil treatments using a novel soil injector of one nozzle with four spouts of spray suspension that are turned upward at an angle of 30° allowing for the uniform spread of fluazinam within a 20 – 30 cm radius and at 0 cm in depth to reach the target zone.

Fluazinam has been also tested to reduce the fungal growth *in vitro* and control white root rot as compared to with the fungicides benomyl, carbendazim and thiophanate methyl on avocado plants under greenhouse conditions. All fungicides were applied eight sequential times at concentrations of 0.1% and 1% (w/v). Fluazinam showed a longer persistence in soil, thus avoiding the continuous applications of other systemic fungicides studied and providing a better control of the disease (López-Herrera and Zea-Bonilla, 2007). Nonetheless, there is no previous study which has reported the application of fluazinam on infested avocado orchards and evaluated the level of *R. necatrix* inoculum in the soil after these treatments.

Integrated control

New environmental trends recommend to reduce the use of chemical compounds for safety food consumption. This reduction on the use of pesticides, particularly fungicides, can be achieved with the integrated control combining different control methods.

Combination of BCA's

Ruano-Rosa et al., (2014) demonstrated that the combination of antagonistic microorganisms such as *Trichoderma* spp. plus rhizobacteria strains (*Bacillus subtilis* and *Pseudomonas* spp.) reduced *in vitro* growth of *R. necatrix*, and additionally, this combination improved significantly the control of white root rot on avocado plants as compared with their respective control.

Combination of BCA plus fungicide

On the other hand, in an additional study on avocado plants carried out by Ruano-Rosa et al. (2017) reduced the effective concentration of the fungicide fluazinam to 100 times lower the field concentration used against white root rot, when combining low concentrations of this fungicide plus *Trichoderma* spp. isolates.

Objectives

The main objective of this Ph.D. Thesis has been obtaining new effective and integrated control methods of avocado white root rot. This has been developed in the following specific objectives and sub-objectives:

1. Study of pathogenicity of *Rosellinia necatrix* isolates.

1.1. Collecting new *Rosellinia necatrix* isolates from avocado orchards, and detection and identification of new mycoviruses (dsRNA) on them.

1.2. Study of the virulence of *R. necatrix* isolates infected by mycoviruses and its relation with the phenomenon of hypovirulence. Horizontal transmission and curing of mycoviruses.

2. Study of integrated control methods of white root rot on avocado plants using the combination of biological antagonistic microorganisms with low concentration of fungicide fluazinam.

2.1. Combination of antagonistic rhizobacteria strains with low concentrations of fluazinam.

2.2. Combination of non-pathogenic *R. necatrix* isolates with low concentrations of fluazinam.

3. Study of chemical control of white root rot on avocado commercial orchards

3.1. Detection and quantification of *R. necatrix* in soil samples from infested avocado orchards.

3.2. Control of avocado white root rot with fluazinam treatments and its evaluation by molecular methods.

4. Study of biological control of white root rot by novel antagonistic fungal isolates (*Entoleuca* sp.).

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Chapter 2: Pathogenicity of *R. necatrix*

Sub-chapter 2.1: Novel, diverse RNA viruses from Mediterranean isolates of the phytopathogenic fungus, *Rosellinia necatrix*: insights into evolutionary biology of fungal viruses

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Novel, diverse RNA viruses from Mediterranean isolates of the phytopathogenic fungus, *Rosellinia necatrix*: insights into evolutionary biology of fungal viruses

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Summary

To reveal mycovirus diversity, we conducted a search of as-yet-unexplored Mediterranean isolates of the phytopathogenic ascomycete *Rosellinia necatrix* for virus infections. Of seventy-nine, eleven fungal isolates tested RNA virus-positive, with many showing coinfections, indicating a virus incidence of 14%, which is slightly lower than that (approximately 20%) previously reported for extensive surveys of over 1000 Japanese *R. necatrix* isolates. All viral sequences were fully or partially characterized by Sanger and next-generation sequencing. These sequences appear to represent isolates of various new species spanning at least 6 established or previously proposed families such as *Partiti-*, *Hypo-*, *Megabirna-*, Yado-kari-, Fusagra- and Fusarividae, as well as a newly proposed family, Megatotiviridae. This observation greatly expands the diversity of *R. necatrix* viruses, because no hypo-, fusagra- or megatotiviruses were previously reported from *R. necatrix*. The sequence analyses showed a rare horizontal gene transfer event of the 2A-like protease domain between a dsRNA (phlegivirus) and a positive-sense, single-stranded RNA virus (hypovirus). Moreover, many of the newly detected viruses showed the closest relation to viruses reported from fungi other than *R. necatrix*, such as *Fusarium* spp., which are sympatric to *R. necatrix*. These combined results imply horizontal virus transfer between these soil-inhabitant fungi.

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Introduction

In the past decades, a number of phytopathogenic and plant-associated fungi have been screened for viruses from a few perspectives such as virological control (biological using viruses) practice and basic virus research. Tested fungi were shown to be infected by viruses at different incidence rates, ranging from a few percentage to over 90% (Hillman *et al.*, 2018). These studies revealed the great diversity of fungal viruses, and provided insights into their evolutionary histories (Xie and Jiang, 2014; Marzano and Domier, 2016; Marzano *et al.*, 2016; Nerva *et al.*, 2016). For example, negative-sense (–), single-stranded (ss) RNA viruses related to known non-segmented and segmented (–)ssRNA viruses were detected from different fungi (Kondo *et al.*, 2013b; Liu *et al.*, 2014; Donaire *et al.*, 2016), while some dsRNA and positive-stranded (+)ssRNA fungal viruses are known to be related to animal/plant viruses (Ghabrial and Suzuki, 2009; Pearson *et al.*, 2009). A gemini-like circular single-stranded (ss) DNA virus termed Sclerotiorum sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV1) was found in an important plant pathogen, *Sclerotinia*

Abstract

To reveal mycovirus diversity, we conducted a search of as-yet-unexplored Mediterranean isolates of the phytopathogenic ascomycete *Rosellinia necatrix* for virus infections. Of seventy-nine, eleven fungal isolates tested RNA virus-positive, with many showing coinfections, indicating a virus incidence of 14%, which is slightly lower than that (approximately 20%) previously reported for extensive surveys of over 1000 Japanese *R. necatrix* isolates. All viral sequences were fully or partially characterized by Sanger and next-generation sequencing. These sequences appear to represent isolates of various new species spanning at least 6 established or previously proposed families such as Partiti-, Hypo-, Megabirna-, Yado-kari-, Fusagra- and Fusarividae, as well as a newly proposed family, Megatotiviridae. This observation greatly expands the diversity of *R. necatrix* viruses, because no hypo-, fusagra- or megatotiviruses were previously reported from *R. necatrix*. The sequence analyses showed a rare horizontal gene transfer event of the 2A-like protease domain between a dsRNA (phlegivirus) and a positive-sense, single-stranded RNA virus (hypovirus). Moreover, many of the newly detected viruses showed the closest relation to viruses reported from fungi other than *R. necatrix*, such as Fusarium spp., which are sympatric to *R. necatrix*. These combined results imply horizontal virus transfer between these soil-inhabitant fungi.

Acknowledgements

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Sub-chapter 2.2: A moderate level of hypovirulence conferred by a hypovirus in the avocado white root rot fungus, *Rosellinia necatrix*

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Abstract

Two isolates of *Rosellinia necatrix* (Rn118-8 and Rn480) have previously obtained from diseased avocado trees in commercial orchards of the coastal area in southern Spain. Rn118-8 and Rn480 have weak virulence on avocado plants, and are infected by Rosellinia necatrix hypovirus 2 (RnHV2). In this work, the possible biological effects of the hypovirus on *R. necatrix* were tested. First, RnHV2 was transmitted from each of Rn118-8 and Rn480 to a highly virulent, mycovirus-free isolate of *R. necatrix* (Rn400) through hyphal anastomosis, using zinc compounds which attenuate the incompatibility reactions. Next, we carried out an analysis of growth rate *in vitro* and a virulence test of these newly infected strains in avocado plants. We obtained five strains of Rn400 infected by RnHV2 after horizontal transmission, all of which showed lower colony growth *in vitro* and lower virulence on avocado plants than virus-free Rn400. These results suggest that *R. necatrix* isolates infected by RnHV2 could be used as novel virocontrol agents to combat avocado white root rot.

Keywords

Anastomosis; mycovirus transmission; *Persea americana*; virocontrol; virulence

Acknowledgements

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Sub-chapter 2.3: A partitivirus of *Rosellinia necatrix* is associated with hypovirulence

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Abstract

Many Mediterranean isolates of *Rosellinia necatrix*, causing root rot in avocado, have previously been screened for viruses. Among them is isolate Rn459 that has been shown to be infected by at least three viruses such as Rosellinia necatrix partitivirus 10 (RnPV10), Rosellinia necatrix fusagravirus 1 (RnFGV1) and a hypovirus. Here, we eliminated RnPV10 by hyphal tipping to examine its effect on colony growth *in vitro* and virulence on avocado plants. RnPV10-free Rn459 (Rn459_PV10F), confirmed to be cured of RnPV10 but retaining the other viruses, manifested a phenotype different from the original Rn459. Colony growth comparison showed that Rn459_PV10F grew faster than the original Rn459 isolate and the virulence on avocado plants of this virus-free strain was higher than the original Rn459 strain. Our findings indicate that RnPV10 contributes to confer hypovirulence on the *R. necatrix* isolates.

Keywords

Curing; hypovirulence; mycovirus; partitivirus; *Persea americana*; *Rosellinia necatrix*; virocontrol

Acknowledgments

This study was supported in part by the Plan Nacional I+D+I Ministerio de Economía y Competitividad (AGL 2014-52518-C2-2-R) Spain. In addition, this research was co-financed by FEDER funds (EU) and by Yomogi Inc. and Grantsin-Aid for Scientific Research on Innovative Areas from the Japanese Ministry of Education, Culture, Sports, Science and Technology (KAKENHI 25252011 and 16H06436, 16H06429 and 16K21723 to N.S. and H. K.).

Chapter 3: Integrated control of avocado white root rot

Sub-chapter 3.1: Combination of low concentrations of fluazinam and antagonistic rhizobacteria to control avocado white root rot

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Combination of low concentrations of fluazinam and antagonistic rhizobacteria to control avocado white root rot

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ABSTRACT

Avocado white root rot is a disease caused by the soil-borne pathogenic ascomycete *Rosellinia necatrix* Prill. In this work, we have carried out different experiments combining the contact fungicide fluazinam with biocontrol rhizobacterial strains of *Pseudomonas chlororaphis* and *Bacillus subtilis*, in order to reduce *in vitro* growth inhibition of *R. necatrix* and to control the disease in avocado plants. When combining fluazinam at a low concentration (0.01 mg L^{-1}) with individual biocontrol bacterial strains, a slight reduction in fungal growth was observed (10 mm^2 for PCL1606; 19 mm^2 for PCL1601 and 11 mm^2 for PCL1608) when compared with the use of the fungicide alone (31 mm^2). From all the assayed rhizobacteria, the combined use of the rhizobacterial strain *Pseudomonas chlororaphis* PCL1606 with fluazinam showed the best fungal inhibition in *in vitro* tests (22–66%). The protective effect against avocado root rot in avocado plants was recorded using a concentration of fluazinam of 0.01%, but its effect was not improved when combined this fungicide with the biocontrol rhizobacteria PCL1606. However, when a lower concentration of fluazinam was tested (0.001%), the combination with PCL1601 or PCL1606 strains resulted in improved control of the disease than when using the fungicide alone with a reduction of 15% and 8% respectively. These results point to the development of an integrated tool to control avocado white root rot by using lower concentrations of fungicide, combined with a suitable biocontrol rhizobacteria, thus obtaining the protective effect on the plant, but reducing the chemical residues and preventing the development of fungal resistance.

1. Introduction

By the late 1970s, avocado (*Persea americana* Mill.) crops were established all along the southern coast area of Spain (López-Herrera and Zea-Bonilla, 2007). The total production of avocado in 2017 was 92,936 tons, produced in an area of 11,812 ha, generating a yield of 7,867 kg/ha and constituting the second most important tropical crop after banana (MAPAMA, 2019).

One of the most important diseases in avocado crops in the Andalusian coastal area of Spain is avocado white root rot (AWRR) caused by the ascomycete *Rosellinia necatrix* Prill. (anamorph: *Dematophora necatrix* Hartig) (López Herrera, 1989). *R. necatrix* is a soil-borne fungus with a worldwide distribution which affects around 170 plant species from 63 genera (ten Hoopen and Krauss, 2006). This pathogen causes important economic losses and plant destruction in many crops, such as Japanese pear and apple in Japan, and in tropical and subtropical species (Arakawa et al., 2002; Sztejnberg and Madar, 1980; ten Hoopen and Krauss, 2006). *R. necatrix* was first detected in 1987 on avocado crops in Spain, since then the disease incidence has increased, and it is considered the most important root disease (López Herrera, 1989). The pathogen has been also reported in other avocado-producing countries such as Mexico, USA (California) and Israel, but with a lower incidence when compared with the Mediterranean area (López-Herrera and Zea-Bonilla, 2007). Symptomatic trees usually show rotten roots with white mycelial fans-like and are characterized by a yellowing of the leaves, which leads to wilt. The death of the tree can occur in a matter of weeks after the appearance of the first foliar symptoms (Ruano-Rosa et al., 2017).

R. necatrix is difficult to control, because this fungus has a wide host range and extensive distribution in the soil, can tolerate both dry conditions and acidic soils and is resistant to many common fungicides (ten Hoopen and Krauss, 2006). In Japan, producers of glasshouse

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Abstract

Avocado white root rot is a disease caused by the soil-borne pathogenic ascomycete *Rosellinia necatrix* Prill. In this work, we have carried out different experiments combining the contact fungicide fluazinam with biocontrol rhizobacterial strains of *Pseudomonas chlororaphis* and *Bacillus subtilis*, in order to reduce *in vitro* growth inhibition of *R. necatrix* and to control the disease in avocado plants. When combining fluazinam at a low concentration (0.01 mg l^{-1}) with individual biocontrol bacterial strains, a slight reduction in fungal growth was observed (10 mm^2 for PCL1606; 19 mm^2 for PCL1601 and 11mm^2 for PCL1608) when compared with the use of the fungicide alone (31mm2). From all the assayed rhizobacteria, the combined use of the rhizobacterial strain *Pseudomonas chlororaphis* PCL1606 with fluazinam showed the best fungal inhibition in in vitro tests (22 – 66%). The protective effect against avocado root rot in avocado plants was recorded using a concentration of fluazinam of 0.01%, but its effect was not improved when combined this fungicide with the biocontrol rhizobacteria PCL1606. However, when a lower concentration of fluazinam was tested (0.001%), the combination with PCL1601 or PCL1606 strains resulted in improved control of the disease than when using the fungicide alone with a reduction of 15% and 8% respectively. These results point to the development of an integrated tool to control avocado white root rot by using lower concentrations of fungicide, combined with a suitable biocontrol rhizobacteria, thus obtaining the protective effect on the plant, but reducing the chemical residues and preventing the development of fungal resistance.

Keywords:

Bacillus spp.; Fluazinam; *Persea americana*; *Pseudomonas* spp.; Rhizobacteria; *Rosellinia necatrix*; White root rot

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Sub-chapter 3.2: Control of avocado white root rot using non-pathogenic *Rosellinia necatrix* isolates combined with low concentrations of fluazinam

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Control of avocado white root rot using non-pathogenic *Rosellinia necatrix* isolates combined with low concentration of fluazinam

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Abstract Different studies to control the *Rosellinia necatrix* growth in vitro and avocado white root rot in vivo were performed, using two non-pathogenic *R. necatrix* isolates (Rn12 and Rn29) combined with fluazinam. The application of non-pathogenic isolates alone showed significant growth reduction as compared to *R. necatrix* pathogenic isolates in a PDA growth medium. The combination of both non-pathogenic isolates plus fluazinam at 0.01 mg l^{-1} significantly increased the reduction of the pathogen growth in vitro. Additionally, the combination of the lowest fluazinam concentration (0.001%) plus Rn12 improved control of the disease as compared when either the same fungicide rate was used alone or this isolate was applied alone on plants, showing a similar response to the use of the highest concentration of fluazinam applied alone (0.01%). In this work, we have obtained a new, integrated biological control method against this avocado disease using non-pathogenic *R. necatrix* isolates combined with low doses of fluazinam.

Keywords Biocontrol · Integrated management · *Persea americana* · Plant disease · Xylariales · Xylariaceae

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Introduction

The avocado (*Persea americana* Mill.) (Laurales: Lauraceae) was established along the coastal area of southern Spain (in the Andalusian provinces of Malaga and Granada) by the end of 1970s (López-Herrera and Zea-Bonilla 2007). Avocado production in Spain was 92,936 tons in 2017, with a total of 11,812 ha under cultivation (FAOSTAT 2019).

White root rot caused by the soil-borne ascomycete *Rosellinia necatrix* Prill. (Xylariales: Xylariaceae) (Anamorph: *Dematophora necatrix* R. Hartig) is one of the most damaging diseases of avocado crops in southern Spain (López-Herrera 1989). It has a world-wide distribution, affecting about 170 plant species and 63 genera (ten Hoopen and Krauss 2006). The incidence of the disease caused by this fungus has progressively increased since the pathogen was detected in 1987 on avocado trees in Spain (López-Herrera 1989) and it is currently considered the major cause of avocado white root rot (AWRR) (López-Herrera et al. 1998). Trees show yellow leaves as symptoms which lead to wilting and roots rotting with the characteristic fan-like white mycelia under the bark. Trees may die in a matter of a few weeks after the appearance of the first foliar symptoms (Ruano-Rosa et al. 2017). This pathogen is difficult to control because it can stand dry conditions and acidic soils, it has a wide range of hosts and extensive distribution in

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Abstract

Different studies to control the *Rosellinia necatrix* growth *in vitro* and avocado white root rot *in vivo* were performed, using two non-pathogenic *R. necatrix* isolates (Rn12 and Rn29) combined with fluazinam. The application of non-pathogenic isolates alone showed significant growth reduction as compared to *R. necatrix* pathogenic isolates in a PDA growth medium. The combination of both non-pathogenic isolates plus fluazinam at 0.01 mg l^{-1} significantly increased the reduction of the pathogen growth *in vitro*. Additionally, the combination of the lowest fluazinam concentration (0.001%) plus Rn12 improved control of the disease as compared when either the same fungicide rate was used alone or this isolate was applied alone on plants, showing a similar response to the use of the highest concentration of fluazinam applied alone (0.01%). In this work, we have obtained a new, integrated biological control method against this avocado disease using non-pathogenic *R. necatrix* isolates combined with low doses of fluazinam.

Keywords

Biocontrol; Integrated management; *Persea Americana;* Plant disease; Xylariales; Xylariaceae

Acknowledgements

This study was supported partly by the Plan Nacional I+D+I Ministerio de Economía y Competitividad (AGL 2014-52518-C2-2-R), Spain. In addition, the research was co-financed by FEDER funds (EU). The authors would like to thank ISK Biosciences Europe S.A. for providing the fungicide fluazinam needed to carry out this work and Professor José María Melero Vara for his assistance on the review.

Chapter 4: Chemical control of avocado white root rot

Sub-chapter 4.1: Improved real-time PCR protocol for the accurate detection and quantification of *Rosellinia necatrix* in avocado orchards

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METHODS PAPER

Improved real-time PCR protocol for the accurate detection and quantification of *Rosellinia necatrix* in avocado orchards

Juan M. Arjona-López · Nieves Capote · Carlos J. López-Herrera

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Abstract
Aims This study aims to develop and validate a new molecular method of detection and quantification of *Rosellinia necatrix* fungus in soil samples and compare it with conventional methods.
Methods We collected 40 soil and root samples (one as negative control) from the soil around avocado trees. The root samples were checked for typical symptoms of *R. necatrix* and the pathogen was identified using the conventional method of plate culture. These results were then corroborated using a new molecular method of detection and quantification of *R. necatrix* in soil samples, and a duplex TaqMan qPCR protocol was designed that included an internal positive control to avoid the detection of false negatives.
Results The molecular detection and quantification method was effective, sensitive and reliable for all 40 soil samples analysed, whereas, with traditional methods, the fungus was isolated in only 24 out of the 26 symptomatic roots from 40 avocado trees sampled. This improved methodology reduces the sample preparation time compared with previous studies, and provides a molecular tool for the reliable and accurate detection and quantification of *R. necatrix* in naturally infested avocado soils.
Conclusions This technique could be applied for the rapid assessment of *R. necatrix* in soils at the pre-planting stage and evaluation of the efficacy of physical, chemical or biological control treatments.

Keywords qPCR · TaqMan · *R. necatrix* · Quantification · Soil

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Introduction

Cultivation of the avocado (*Persea americana* Mill.) was established on the southern coast of Andalusia (Spain) in the late 1970s and it is an irrigated crop that provides an alternative to the traditional non-irrigated crops such as almond, olive and grapevine (López-Herrera and Zea-Bonilla 2007). The total Spanish avocado production in 2017 was 92,936 tons, produced in 11,812 ha and generating a yield of 7,867 kg/ha, and it is the second most important tropical fruit in Spain after the banana. The region of Andalusia is the highest producer of this crop in Spain, with a total production of 79,760 tons in 2017 (MAPAMA 2019).

Avocado white root rot (AWRR) is caused by the ascomycete *Rosellinia necatrix* Prill. (anamorph: *Dematophora necatrix*). This fungus is a polyphagous pathogen (170 plant species in 63 genera) with a world-wide distribution (ten Hoopen and Krauss 2006), and it

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Abstract

Aims

This study aims to develop and validate a new molecular method of detection and quantification of *Rosellinia necatrix* fungus in soil samples and compare it with conventional methods.

Methods

We collected 40 soil and root samples (one as negative control) from the soil around avocado trees. The root samples were checked for typical symptoms of *R. necatrix* and the pathogen was identified using the conventional method of plate culture. These results were then corroborated using a new molecular method of detection and quantification of *R. necatrix* in soil samples, and a duplex TaqMan qPCR protocol was designed that included an internal positive control to avoid the detection of false negatives.

Results

The molecular detection and quantification method was effective, sensitive and reliable for all 40 soil samples analysed, whereas, with traditional methods, the fungus was isolated in only 24 out of the 26 symptomatic roots from 40 avocado trees sampled. This improved methodology reduces the sample preparation time compared with previous studies, and provides a molecular tool for the reliable and accurate detection and quantification of *R. necatrix* in naturally infested avocado soils.

Conclusions

This technique could be applied for the rapid assessment of *R. necatrix* in soils at the preplanting stage and evaluation of the efficacy of physical, chemical or biological control treatments.

Keywords

qPCR; TaqMan; *R. necatrix*; Quantification; Soil

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Sub-chapter 4.2: Control of avocado white root rot by chemical treatments with Fluazinam in avocado orchards

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Abstract

Rosellinia necatrix Prill. is the causal soil-borne agent of avocado white root rot disease. This work proposes a new method of treatment with fungicide to combat this disease, aimed to obtain a quantitative assessment of the evolution of fungal inoculum in soil samples. A total of 39 avocado trees were selected from five commercial orchards affected by *R. necatrix*. Root samples were collected to recover *R. necatrix* isolates. The fluazinam treatments were applied around each tree by soil injection, and soil samples were collected before and after each fungicide application to analyze the evolution of the fungus by qPCR. In addition, two samplings of avocado fruits during two harvesting seasons were performed to measure the chemical residues. All 24 *R. necatrix* isolates taken from avocado roots were pathogenic. The qPCR quantification results showed that the percentage of fungal inoculum in the soil was significantly lower after the first fluazinam treatment compared to that before any fungicide had been applied. Furthermore, soil from non-treated avocado trees did not show any significant differences in the concentration of fungal inoculum. The chemical analysis of the fruits did not show residues. These results provide growers with a new method to control this soil-borne pathogen in crops.

Keywords

Disease control; Fluazinam; fungicide residues; qPCR

Acknowledgements

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Chapter 5: Biological control of avocado white root rot

Sub-chapter 5.1: *Entoleuca* sp. infected by mycoviruses as potential biocontrol agents of avocado white root rot

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Abstract

Rosellinia necatrix Prill. is the causal agent of white root rot diseases, which cause major losses in avocado crops in Spain. To study the biocontrol of this disease, 31 antagonist isolates were collected from the avocado rhizosphere and identified as *Entoleuca* sp. Studies were carried out in mycovirus detection, *in vitro* optimal growth temperature, mycelial compatibility groups of these isolates and their inhibition of *R. necatrix* growth. The pathogenicity of this antagonist and its biocontrol of white root rot on avocado plants were also studied. In this way, the presence of mycoviruses was detected in all 31 isolates studied. The optimal growth temperature for almost all the isolates (> 80%) was 25 °C. Dual culture showed different responses, with some isolates compatible and others incompatible with each other, showing inhibition halos and black lines. 25 of the 31 isolates showed significant growth inhibition to one *R. necatrix* isolate in *in vitro* conditions. In addition, none of these *Entoleuca* sp. isolates produced any symptoms on inoculated avocado plants and in 24 of them, the disease symptoms of avocado white root rot were reduced, with significant differences among them when they were used as biocontrol agents. In this way, new virus-carrying *Entoleuca* sp. isolates were obtained, which are antagonistic fungi capable of controlling avocado white root rot.

Keywords

Avocado; biocontrol; dsRNA; mycoviruses; *Persea americana*; *Rosellinia necatrix*

Acknowledgements

This study was partly supported by the Plan Nacional I+D+I Ministerio de Economía y Competitividad (AGL 2014-52518-C2-2-R) Spain. In addition, this research was co-financed by FEDER funds (EU). The authors would like to thank TROPS for their technical support, especially in the location of isolates in commercial avocado orchards.

Chapter 6: Conclusions

Conclusions

From all chapters of this Doctoral Thesis different conclusions have obtained which are detailed in the following section:

1. Mycoviruses infecting nine *R. necatrix* isolates (14%) of 62 isolates, have been detected and identified, belonging all of these viruses to new viral species from previously proposed families, as well as, a new proposed family, Megatoviridae.
2. Mycoviruses horizontal transmission has been successfully carried out through hyphal anastomosis using zinc compounds to reduce the incompatibility between isolates of *R. necatrix.* The hypovirus RnHV2 has been transmitted from two different low virulent *R. necatrix* isolates to a virus-free and highly pathogenic isolate. Furthermore, the colony growth *in vitro* of positive transmitted isolate was reduced as well as its virulence on avocado plants as compared with the same virus-free strain.
3. The partitivirus RnPV10 from the *R. necatrix* strain Rn459 was positively cured through hyphal tip isolation. This cured isolate showed a higher growth *in vitro* and a higher virulence on avocado plants compared with the same isolate but carrying RnPV10.
4. The combination of rhizobacteria strains PCL1601 and PCL1606 with the lowest effective concentration (0.001%) of fluazinam improved the control of white root rot on avocado plants as compared with the single application of each bacterial strain or of fungicide at this concentration.
5. The combination of non-pathogenic *R. necatrix* Rn12 with the lowest effective concentration (0.001%) of fluazinam improved the control of white root rot on avocado plants when compared with the single application of Rn12 or the fungicide at that concentration.
6. An optimized and reliable molecular method of *R. necatrix* of detection and quantification from soil samples has been performed. This new technique is helpful to prevent the infection of new planting tree crops on soils with a high risk of white root rot and allows the quantification of fungal inoculum of *R. necatrix* after different treatments.
7. The treatments of fluazinam to control white root rot on avocado commercial orchards through soil injections were very effective reducing the fungus soil inoculum density and allowing an effective reduction of this disease.
8. *Entoleuca* sp. isolates have been confirmed as novel biocontrol agents of *R. necatrix in vitro* and of avocado white root rot.